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Lucille M. Begalla
(TYPED OR PRINTED NAME OF PERSON MAILING PAPER)

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Lucille M. Begalla
(TYPED OR PRINTED NAME OF PERSON MAILING PAPER)
Lucille M. Begalla
(SIGNATURE OF PERSON MAILING PAPER OR FEE)

for

UNITED STATES LETTERS PATENT

on

HIGH THROUGHPUT SCREENING FOR NOVEL ENZYMES

by

JAY M. SHORT

and

MARTIN KELLER

Sheets of Drawings: 16

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Lisa A. Haile, Ph.D.
Gray Cary Ware & Freidenrich
4365 Executive Drive, Suite 1600
San Diego, California 92121-2189

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HIGH THROUGHPUT SCREENING FOR NOVEL ENZYMES

[0001] This application is a divisional of United States Patent Application No. 09/636,778, filed August 11, 2000, which is a continuation of United States Patent Application No. 09/098,206, filed June 16, 1998, ^{now U.S. Patent No. 6,174,673} continuation-in-part of U.S. Application Serial No. 08/876,276, filed June 16, 1997, now abandoned.

Field of the Invention

[0002] The present invention relates generally to the identification of new bioactive molecules and particularly to methods for recovering such molecules by co-encapsulation and fluorescence activated cell sorting (FACS).

Background of the Invention

[0003] There is a critical need in the chemical industry for efficient catalysts for the practical synthesis of optically pure materials; enzymes can provide the optimal solution. All classes of molecules and compounds that are utilized in both established and emerging chemical, pharmaceutical, textile, food and feed, detergent markets must meet stringent economical and environmental standards. The synthesis of polymers, pharmaceuticals, natural products and agrochemicals is often hampered by expensive processes which produce harmful byproducts and which suffer from low enantioselectivity (Faber, 1995; Tonkovich and Gerber, U.S. Dept of Energy study, 1995). Enzymes have a number of remarkable advantages which can overcome these problems in catalysis: they act on single functional groups, they distinguish between similar functional groups on a single molecule, and they distinguish between enantiomers. Moreover, they are biodegradable and function at very low mole fractions in reaction mixtures. Because of their chemo-, regio- and stereospecificity, enzymes present a unique opportunity to optimally achieve desired selective transformations. These are often extremely difficult to duplicate chemically, especially in single-step reactions. The elimination of the need for protection groups, selectivity, the ability to carry out multi-step transformations in a single reaction

vessel, along with the concomitant reduction in environmental burden, has led to the increased demand for enzymes in chemical and pharmaceutical industries (Faber, 1995). Enzyme-based processes have been gradually replacing many conventional chemical-based methods (Wrotnowski, 1997). A current limitation to more widespread industrial use is primarily due to the relatively small number of commercially available enzymes. Only ~300 enzymes (excluding DNA modifying enzymes) are at present commercially available from the > 3000 non DNA-modifying enzyme activities thus far described.

[0004] The use of enzymes for technological applications also may require performance under demanding industrial conditions. This includes activities in environments or on substrates for which the currently known arsenal of enzymes was not evolutionarily selected. Enzymes have evolved by selective pressure to perform very specific biological functions within the milieu of a living organism, under conditions of mild temperature, pH and salt concentration. For the most part, the non-DNA modifying enzyme activities thus far described (Enzyme Nomenclature, 1992) have been isolated from mesophilic organisms, which represent a very small fraction of the available phylogenetic diversity (Amann et al., 1995). The dynamic field of biocatalysis takes on a new dimension with the help of enzymes isolated from microorganisms that thrive in extreme environments. Such enzymes must function at temperatures above 100°C in terrestrial hot springs and deep sea thermal vents, at temperatures below 0°C in arctic waters, in the saturated salt environment of the Dead Sea, at pH values around 0 in coal deposits and geothermal sulfur-rich springs, or at pH values greater than 11 in sewage sludge (Adams and Kelly, 1995). Enzymes obtained from these extremophilic organisms open a new field in biocatalysis.

while #2 only acts on long chain substrates in addition to a huge difference in the optimal reaction temperature. These results suggest that more diverse enzymes fulfilling the need for new biocatalysts can be found by screening biodiversity. Substrates upon which enzymes act are herein defined as bioactive substrates.

[0006] Furthermore, virtually all of the enzymes known so far have come from cultured organisms, mostly bacteria and more recently archaea (Enzyme Nomenclature, 1992). Traditional enzyme discovery programs rely solely on cultured microorganisms for their screening programs and are thus only accessing a small fraction of natural diversity. Several recent studies have estimated that only a small percentage, conservatively less than 1%, of organisms present in the natural environment have been cultured (see Table I, Amann et al., 1995, Barns et. al 1994, Torvsik, 1990). For example, Norman Pace's laboratory recently reported intensive untapped diversity in water and sediment samples from the "Obsidian Pool" in Yellowstone National Park, a spring which has been studied since the early 1960's by microbiologists (Barns, 1994). Amplification and cloning of 16S rRNA encoding sequences revealed mostly unique sequences with little or no representation of the organisms which had previously been cultured from this pool. This suggests substantial diversity of archaea with so far unknown morphological, physiological and biochemical features which may be useful in industrial processes. David Ward's laboratory in Bozmen, Montana has performed similar studies on the cyanobacterial mat of Octopus Spring in Yellowstone Park and came to the same conclusion, namely, tremendous uncultured diversity exists (Bateson et al., 1989). Giovannoni *et al.* (1990) reported similar results using bacterioplankton collected in the Sargasso Sea while Torsvik *et al.* (1990) have shown by DNA reassociation kinetics that there is considerable diversity in soil samples. Hence, this vast majority of microorganisms represents an untapped resource for the discovery of novel biocatalysts. In order to access this potential catalytic diversity, recombinant screening approaches are required.

[0007] The discovery of novel bioactive molecules other than enzymes is also afforded by the present invention. For instance, antibiotics, antivirals, antitumor agents and regulatory proteins can be discovered utilizing the present invention.

[0008] Bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred to as "gene clusters," on a single chromosome and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. The gene cluster, the promoter, and additional sequences that function in regulation altogether are referred to as an "operon" and can include up to 20 or more genes, usually from 2 to 6 genes. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function.

[0009] Some gene families consist of one or more identical members. Clustering is a prerequisite for maintaining identity between genes, although clustered genes are not necessarily identical. Gene clusters range from extremes where a duplication is generated of adjacent related genes to cases where hundreds of identical genes lie in a tandem array. Sometimes no significance is discernable in a repetition of a particular gene. A principal example of this is the expressed duplicate insulin genes in some species, whereas a single insulin gene is adequate in other mammalian species.

[0010] It is important to further research gene clusters and the extent to which the full length of the cluster is necessary for the expression of the proteins resulting therefrom. Gene clusters undergo continual reorganization and, thus, the ability to create heterogeneous libraries of gene clusters from, for example, bacterial or other prokaryote sources is valuable in determining sources of novel proteins, particularly including enzymes such as, for example, the polyketide synthases that are responsible for the synthesis of polyketides having a vast array of useful activities. As indicated,

[illegible]

[0012] Of particular interest are cellular “switches” known as receptors which interact with a variety of biomolecules, such as hormones, growth factors, and neurotransmitters, to mediate the transduction of an “external” cellular signaling event into an “internal” cellular signal. External signaling events include the binding of a ligand to the receptor, and internal events include the modulation of a pathway in the cytoplasm or nucleus involved in the growth, metabolism or apoptosis of the cell. Internal events also include the inhibition or activation of transcription of certain nucleic acid sequences, resulting in the increase or decrease in the production or presence of certain molecules (such as nucleic acid, proteins, and/or other molecules affected by this increase or decrease in transcription). Drugs to cure disease or alleviate its symptoms can activate or block any of these events to achieve a desired pharmaceutical effect.

[illegible][illegible][illegible][illegible]

prescription drugs work by somehow interacting with G protein-coupled receptors. However, these drugs were developed using classical medicinal chemistry and without a knowledge of the molecular mechanism of action. A more efficient drug discovery program could be deployed by targeting individual receptors and making use of information on gene sequence and biological function to develop effective therapeutics. The present invention allows one to, for example, study molecules which affect the interaction of G proteins with receptors, or of ligands with receptors.

[0017] Several groups have reported cells which express mammalian G proteins or subunits thereof, along with mammalian receptors which interact with these molecules. For example, WO92/05244 (April 2, 1992) describes a transformed yeast cell which is incapable of producing a yeast G protein α subunit, but which has been engineered to produce both a mammalian G protein α subunit and a mammalian receptor which interacts with the subunit. The authors found that a modified version of a specific mammalian receptor integrated into the membrane of the cell, as shown by studies of the ability of isolated membranes to interact properly with various known agonists and antagonists of the receptor. Ligand binding resulted in G protein-mediated signal transduction.

[0018] Another group has described the functional expression of a mammalian adenylyl cyclase in yeast, and the use of the engineered yeast cells in identifying potential inhibitors or activators of the mammalian adenylyl cyclase (WO 95/30012). Adenylyl cyclase is among the best studied of the effector molecules which function in mammalian cells in response to activated G proteins. "Activators" of adenylyl cyclase cause the enzyme to become more active, elevating the cAMP signal of the yeast cell to a detectable degree. "Inhibitors" cause the cyclase to become less active, reducing the cAMP signal to a detectable degree. The method describes the use of the engineered yeast cells to screen for drugs which activate or inhibit adenylyl cyclase by their action on G protein-coupled receptors.

[0022] Flow cytometry has also been used in cloning and selection of variants from existing cell clones. This selection, however, has required stains that diffuse through cells passively, rapidly and irreversibly, with no toxic effects or other influences on metabolic or physiological processes. Since, typically, flow sorting has been used to study animal cell culture performance, physiological state of cells, and the cell cycle, one goal of cell sorting has been to keep the cells viable during and after sorting.

[0023] There currently are no reports in the literature of screening and discovery of recombinant enzymes in *E. coli* expression libraries by fluorescence activated cell sorting of single cells. Furthermore there are no reports of recovering DNA encoding bioactivities screened by expression screening in *E. coli* using a FACS machine. The present invention provides these methods to allow the extremely rapid screening of viable or non-viable cells to recover desirable activities and the nucleic acid encoding those activities.

[0024] A limited number of papers describing various applications of flow cytometry in the field of microbiology and sorting of fluorescence activated microorganisms have, however, been published (Davey and Kell, 1996). Fluorescence and other forms of staining have been employed for microbial discrimination and identification, and in the analysis of the interaction of drugs and antibiotics with microbial cells. Flow cytometry has been used in aquatic biology, where autofluorescence of photosynthetic pigments are used in the identification of algae or DNA stains are used to quantify and count marine populations (Davey and Kell, 1996). Thus, Diaper and Edwards used flow cytometry to detect viable bacteria after staining with a range of fluorogenic esters including fluorescein diacetate (FDA) derivatives and CemChrome B, a proprietary stain sold commercially for the detection of viable bacteria in suspension (Diaper and Edwards, 1994). Labeled antibodies and oligonucleotide probes have also been used for these purposes.

[0025] Papers have also been published describing the application of flow cytometry to the detection of native and recombinant enzymatic activities in eukaryotes. Betz et al. studied native (non-recombinant) lipase production by the eukaryote, *Rhizopus arrhizus* with flow cytometry. They found that spore suspensions of the mold were heterogeneous as judged by light-scattering data obtained with excitation at 633 nm, and they sorted clones of the subpopulations into the wells of microtiter plates. After germination and growth, lipase production was automatically assayed (turbidimetrically) in the microtiter plates, and a representative set of the most active were reisolated, cultured, and assayed conventionally (Betz et al., 1984).

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[0026] Scienc *et al.* have reported a flow cytometric method for detecting cloned β -galactosidase activity in the eukaryotic organism, *S. cerevisiae*. The ability of flow cytometry to make measurements on single cells means that individual cells with high levels of expression (*e.g.*, due to gene amplification or higher plasmid copy number) could be detected. In the method reported, a non-fluorescent compound β -naphthol- β -galactopyranoside) is cleaved by β -galactosidase and the liberated naphthol is trapped to form an insoluble fluorescent product. The insolubility of the fluorescent product is of great importance here to prevent its diffusion from the cell. Such diffusion would not only lead to an underestimation of β -galactosidase activity in highly active cells but could also lead to an overestimation of enzyme activity in inactive cells or those with low activity, as they may take up the leaked fluorescent compound, thus reducing the apparent heterogeneity of the population.

[0027] One group has described the use of a FACS machine in an assay detecting fusion proteins expressed from a specialized transducing bacteriophage in the prokaryote *Bacillus subtilis* (Chung, et.al., J. of Bacteriology, Apr. 1994, p. 1977-1984; Chung, et.al., Biotechnology and Bioengineering, Vol. 47, pp. 234-242 (1995)). This group monitored the expression of a lacZ gene (encodes β -galactosidase) fused to the sporulation loci in subtilis (*spo*). The technique used to monitor β -galactosidase expression from *spo-lacZ* fusions in single cells involved taking samples from a sporulating culture, staining them with a commercially

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available fluorogenic substrate for β -galactosidase called C8-FDG, and quantitatively analyzing fluorescence in single cells by flow cytometry. In this study, the flow cytometer was used as a detector to screen for the presence of the *spo* gene during the development of the cells. The device was not used to screen and recover positive cells from a gene expression library or nucleic acid for the purpose of discovery.

[0028] Another group has utilized flow cytometry to distinguish between the developmental stages of the delta-proteobacteria *Myxococcus xanthus* (F. Russo-Marie, et.al., PNAS, Vol. 90, pp.8194-8198, September 1993). As in the previously described study, this study employed the capabilities of the FACS machine to detect and distinguish genotypically identical cells in different development regulatory states. The screening of an enzymatic activity was used in this study as an indirect measure of developmental changes.

[0029] The lacZ gene from E.coli is often used as a reporter gene in studies of gene expression regulation, such as those to determine promoter efficiency, the effects of *trans*-acting factors, and the effects of other regulatory elements in bacterial, yeast, and animal cells. Using a chromogenic substrate, such as ONPG (*o*-nitrophenyl-(*D*-galactopyranoside), one can measure expression of β -galactosidase in cell cultures; but it is not possible to monitor expression in individual cells and to analyze the heterogeneity of expression in cell populations. The use of fluorogenic substrates, however, makes it possible to determine β -galactosidase activity in a large number of individual cells by means of flow cytometry. This type of determination can be more informative with regard to the physiology of the cells, since gene expression can be correlated with the stage in the mitotic cycle or the viability under certain conditions. In 1994, Plovins *et al.*, reported the use of fluorescein-Di- β -*D*-galactopyranoside (FDG) and C₁₂-FDG as substrates for β -galactosidase detection in animal, bacterial, and yeast cells. This study compared the two molecules as substrates for β -galactosidase, and concluded that FDG is a better substrate for β -galactosidase detection by flow cytometry in bacterial cells. The screening performed in this study was for the comparison of the

two substrates. The detection capabilities of a FACS machine were employed to perform the study on viable bacterial cells.

[0030] Cells with chromogenic or fluorogenic substrates yield colored and fluorescent products, respectively. Previously, it had been thought that the flow cytometry-fluorescence activated cell sorter approaches could be of benefit only for the analysis of cells that contain intracellularly, or are normally physically associated with, the enzymatic activity of small molecule of interest. On this basis, one could only use fluorogenic reagents which could penetrate the cell and which are thus potentially cytotoxic. To avoid clumping of heterogeneous cells, it is desirable in flow cytometry to analyze only individual cells, and this could limit the sensitivity and therefore the concentration of target molecules that can be sensed. Weaver and his colleagues at MIT and others have developed the use of gel microdroplets containing (physically) single cells which can take up nutrients, secret products, and grow to form colonies. The diffusional properties of gel microdroplets may be made such that sufficient extracellular product remains associated with each individual gel microdroplet, so as to permit flow cytometric analysis and cell sorting on the basis of concentration of secreted molecule within each microdroplet. Beads have also been used to isolate mutants growing at different rates, and to analyze antibody secretion by hybridoma cells and the nutrient sensitivity of hybridoma cells. The gel microdroplet method has also been applied to the rapid analysis of mycobacterial growth and its inhibition by antibiotics.

[0031] The gel microdroplet technology has had significance in amplifying the signals available in flow cytometric analysis, and in permitting the screening of microbial strains in strain improvement programs for biotechnology. Wittrup *et al.*, (Biotechnol. Bioeng. (1993) 42:351-356) developed a microencapsulation selection method which allows the rapid and quantitative screening of $>10^6$ yeast cells for enhanced secretion of *Aspergillus awamori* glucoamylase. The method provides a 400-fold single-pass enrichment for high-secretion mutants.

[0032] Gel microdroplet or other related technologies can be used in the present invention to localize as well as amplify signals in the high throughput screening of recombinant libraries. Cell viability during the screening is not an issue or concern since nucleic acid can be recovered from the microdroplet.

[0033] Different types of encapsulation strategies and compounds or polymers can be used with the present invention. For instance, high temperature agaroses can be employed for making microdroplets stable at high temperatures, allowing stable encapsulation of cells subsequent to heat kill steps utilized to remove all background activities when screening for thermostable bioactivities.

[0034] There are several hurdles which must be overcome when attempting to detect and sort *E. coli* expressing recombinant enzymes, and recover encoding nucleic acids. FACS systems have typically been based on eukaryotic separations and have not been refined to accurately sort single *E. coli* cells; the low forward and sideward scatter of small particles like *E. coli*, reduces the ability of accurate sorting; enzyme substrates typically used in automated screening approaches, such as umbelliferyl based substrates, diffuse out of *E. coli* at rates which interfere with quantitation. Further, recovery of very small amounts of DNA from sorted organisms can be problematic. The present invention addresses and overcomes these hurdles and offers a novel screening approach.

Summary of the Invention

[0035] The present invention adapts traditional eukaryotic flow cytometry cell sorting systems to high throughput screening for expression clones in prokaryotes. In the present invention, expression libraries derived from DNA, primarily DNA directly isolated from the environment, are screened very rapidly for bioactivities of interest utilizing fluorescence activated cell sorting. These libraries can contain greater than 10^8 members and can represent single organisms or can represent the genomes of over 100 different microorganisms, species or subspecies.

[0037] More particularly, the invention provides a process for identifying clones having a specified activity of interest by (i) generating one or more expression libraries made to contain nucleic acid directly or indirectly isolated from the environment; (ii) exposing said libraries to a particular substrate or substrates of interest; and (iii) screening said exposed libraries utilizing a high throughput cell analyzer, preferably a fluorescence activated cell sorter, to identify clones which react with the substrate or substrates.

[0038] In another aspect, the invention also provides a process for identifying clones having a specified activity of interest by (i) generating one or more expression libraries derived from nucleic acid directly or indirectly isolated from the environment; and (ii) screening said exposed libraries utilizing an assay requiring a binding event or the covalent modification of a target, and a high throughput cell analyzer, preferably a fluorescence activated cell sorter, to identify positive clones.

[0039] The invention further provides a method of screening for an agent that modulates the activity of a target protein or other cell component (*e.g.*, nucleic acid), wherein the target and a selectable marker are expressed by a recombinant cell, by co-encapsulating the agent in a micro-environment with the recombinant cell expressing the target and detectable marker and detecting the effect of the agent on the activity of the target cell component.

[0040] In another embodiment, the invention provides a method for enriching for target DNA sequences containing at least a partial coding region for at least one specified activity in a DNA sample by co-encapsulating a mixture of target DNA

obtained from a mixture of organisms with a mixture of DNA probes including a detectable marker and at least a portion of a DNA sequence encoding at least one enzyme having a specified enzyme activity and a detectable marker; incubating the co-encapsulated mixture under such conditions and for such time as to allow hybridization of complementary sequences and screening for the target DNA. Optionally the method further comprises transforming host cells with recovered target DNA to produce an expression library of a plurality of clones.


[0041] The invention further provides a method of screening for an agent that modulates the interaction of a first test protein linked to a DNA binding moiety and a second test protein linked to a transcriptional activation moiety by co-encapsulating the agent with the first test protein and second test protein in a suitable microenvironment and determining the ability of the agent to modulate the interaction of the first test protein linked to a DNA binding moiety with the second test protein covalently linked to a transcriptional activation moiety, wherein the agent enhances or inhibits the expression of a detectable protein. Preferably, screening is by FACS analysis.

Brief Description of the Drawings

[0042] Figure 1 illustrates the substrate spectrum fingerprints and optimum reaction temperatures of five of novel esterases showing the diversity in these enzymes. EST# indicates the different enzyme; the temperatures indicate the optimal growth temperatures for the organisms from which the esterases were isolated; "E" indicates the relative activity of each esterase enzyme on each of the given substrates indicated (Hepanoate being the reference).

[0043] Figure 2 illustrates the cloning of DNA fragments prepared by random cleavage of target DNA to generate a representative library as described in Example 1.

[0044] Figure 3 shows a statistical analysis of the total number of clones to be tested (e.g. the number of genome equivalents). Assuming that mechanical shearing and gradient purification results in normal distribution of DNA fragment sizes with a mean of 4.5 kbp and variance of 1 kbp, the fraction represented of all possible 1 kbp sequences in a 1.8 Mbp genome is plotted in Figure 3 as a function of increasing genome equivalents.


[0045] Figure 4 illustrates the protocol used in the cell sorting method of the invention to screen for recombinant enzymes, in this case using a (library excised into *E. coli*). The expression clones of interest are isolated by sorting. The procedure is described in detail in Examples 1,3 and 4.

[0046] Figure 5 shows β -galactosidase clones stained with three different substrates: fluorescein-di- β -D-galactopyranoside (FDG), C12-fluorescein-di- β -D-galactopyranoside (C12FDG), chloromethyl-fluorescein-di- β -D-galactopyranoside (CMFDG). *E.coli* expressing β -galactosidase from *Sulfolobus sulfotaricus* species was grown overnight. Cells were centrifuged and substrate was loaded with deionized water. After five (5) minutes cells were centrifuged and transferred into HEPES buffer and heated to 70°C for thirty (30) minutes. Cells were spotted onto a slide and exposed to UV light. This illustrates the results of the experiments described in Example 3.

[0047] Figure 6 shows a microtiter plate where *E.coli* cells sorted in accordance with the invention are dispensed, one cell per well and grown up as clones which are then stained with fluorescein-di- β -D-galactopyranoside (FDG) (10mM). This illustrates the results of the experiments described in Example 5.

[0048] Figure 7 shows the principle type of fluorescence enzyme assay of deacylation.

[0049] Figure 8 shows staining of β -galactosidase clones from the hyperthermophilic archaean *Sulfolobus solfataricus* expressed in *E.coli* using C_{12} -FDG as enzyme substrate.

[0050] Figure 9 shows the synthesis of 5-dodecanoyl-aminofluorescein-di-dodecanoic acid.

[0051] Figure 10 shows Rhodamine protease substrate. Figure 11 shows a compound and process that can be used in the detection of monooxygenases.

[0052] Figure 12 is a schematic illustration of combinatorial enzyme development using directed evolution.

[0053] Figure 13 is a schematic illustration showing bypassing barriers to directed evolution.

[0054] Figure 14 depicts a co-encapsulation assay for a novel bioactive screen. Cells containing large insert library clones are coencapsulated with a eukaryotic cell containing a receptor. Binding of the receptor by a small molecule expressed from the library ultimately yields expression of a GFP reporter molecule. Encapsulation can occur in a variety of means, including gel microdroplets, liposomes, and ghost cells. Cells are screened via high throughput screening on a fluorescence analyzer.

[0055] Figure 15 depicts co-encapsulation of test organisms with pathway clones and sorting based on assays for bioactive expression of clones, such as affects on growth rates of test organisms. In this figure, sorting occurs on a FACS machine.

[0056] Figure 16 depicts micrographs of *Streptomyces* strains. The picture on the left represents *Streptomyces lividans* mycelia, and the right depicts unicells of another species of *Streptomyces* which forms unicells (100X objective phase contrast; taken from an Olympus microscope).

The first part of the paper is devoted to the study of the asymptotic behavior of the solutions of the system (1.1) as $\epsilon \rightarrow 0$. In the second part, we study the asymptotic behavior of the solutions of the system (1.1) as $\epsilon \rightarrow 0$. In the third part, we study the asymptotic behavior of the solutions of the system (1.1) as $\epsilon \rightarrow 0$.

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culture processes. FACS has also been utilized to monitor production of foreign proteins in both eukaryotes and prokaryotes to study, for example, differential gene expression, etc. The detection and counting capabilities of the FACS system have been applied in these examples. However, FACS has never previously been employed in a discovery process to screen for and recover bioactivities in prokaryotes. Furthermore, the present invention does not require cells to survive, as do previously described technologies, since the desired nucleic acid (recombinant clones) can be obtained from alive or dead cells. The cells only need to be viable long enough to produce the compound to be detected, and can thereafter be either viable or non-viable cells so long as the expressed biomolecule remains active. The present invention also solves problems that would have been associated with detection and sorting of *E. coli* expressing recombinant enzymes, and recovering encoding nucleic acids. Additionally, the present invention includes within its embodiments any apparatus capable of detecting ^{fluorescent} fluorescent wavelengths associated with biological material, such apparatus are defined herein as fluorescent analyzers (one example of which is a FACS).

[0061] The use of a culture-independent approach to directly clone genes encoding novel enzymes from environmental samples allows one to access untapped resources of biodiversity. The approach is based on the construction of "environmental libraries" which represent the collective genomes of naturally occurring organisms archived in cloning vectors that can be propagated in suitable prokaryotic hosts. Because the cloned DNA is initially extracted directly from environmental samples, the libraries are not limited to the small fraction of prokaryotes that can be grown in pure culture. Additionally, a normalization of the environmental DNA present in these samples could allow more equal representation of the DNA from all of the species present in the original sample. This can dramatically increase the efficiency of finding interesting genes from minor constituents of the sample which may be under-represented by several orders of magnitude compared to the dominant species.

[illegible][illegible][illegible]

[0065] The method of the present invention begins with the construction of gene libraries which represent the collective genomes of naturally occurring organisms archived in cloning vectors that can be propagated in suitable prokaryotic hosts. The microorganisms from which the libraries may be prepared include prokaryotic microorganisms, such as Eubacteria and Archaeobacteria, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. Libraries may be produced from environmental samples in which case DNA may be recovered without culturing of an organism or the DNA may be recovered from a cultured organism is described and exemplified in detail in co-pending, commonly assigned U.S. Serial No. 08/657,409, filed June 6, 1996, which is incorporated herein by reference. Such microorganisms may be extremophiles, such as hyperthermophiles, psychrophiles, psychrotrophs, halophiles, alkalophiles, acidophiles, *etc.*

[0066] Sources of microorganism DNA as a starting material library from which target DNA is obtained are particularly contemplated to include environmental samples, such as microbial samples obtained from Arctic and Antarctic ice, water or permafrost sources, materials of volcanic origin, materials from soil or plant sources in tropical areas, etc. Thus, for example, genomic DNA may be recovered from either a culturable or non-culturable organism and employed to produce an appropriate recombinant expression library for subsequent determination of enzyme or other biological activity. Prokaryotic expression libraries created from such starting material which includes DNA from more than one species are defined herein as multispecific libraries.

[0067] In one embodiment, viable or non-viable cells isolated from the environment are, prior to the isolation of nucleic acid for generation of the expression gene library, FACS sorted to separate prokaryotic cells from the sample based on, for instance, DNA or AT/GC content of the cells. Various dyes or stains well known in the art, for example those described in "Practical Flow Cytometry", 1995 Wiley-Liss, Inc., Howard M. Shapiro, M.D., are used to intercalate or associate with nucleic acid of cells, and cells are separated on the FACS based on relative DNA content or

AT/GC DNA content in the cells. Other criteria can also be used to separate prokaryotic cells from the sample, as well. DNA is then isolated from the cells and used for the generation of expression gene libraries, which are then screened using the FACS for activities of interest.

[0068] Alternatively, the nucleic acid is isolated directly from the environment and is, prior to generation of the gene library, sorted based on DNA or AT/GC content. DNA isolated directly from the environment, is used intact, randomly sheared or digested to general fragmented DNA. The DNA is then bound to an intercalating agent as described above, and separated on the analyzer based on relative base content to isolate DNA of interest. Sorted DNA is then used for the generation of gene libraries, which are then screened using the analyzer for activities of interest.

[0069] The present invention can further optimize methods for isolation of activities of interest from a variety of sources, including consortias of microorganisms, primary enrichments, and environmental "uncultivated" samples, to make libraries which have been "normalized" in their representation of the genome populations in the original samples. and to screen these libraries for enzyme and other bioactivities. Libraries with equivalent representation of genomes from microbes that can differ vastly in abundance in natural populations are generated and screened. This "normalization" approach reduces the redundancy of clones from abundant species and increases the representation of clones from rare species. These normalized libraries allow for greater screening efficiency resulting in the identification of cells encoding novel biological catalysts.

[0070] One embodiment for forming a normalized library from an environmental sample begins with the isolation of nucleic acid from the sample. This nucleic acid can then be fractionated prior to normalization to increase the chances of cloning DNA from minor species from the pool of organisms sampled. DNA can be fractionated using a density centrifugation technique, such as a cesium-chloride gradient. When an intercalating agent, such as bis-benzimide is employed to change

cultivated organisms. DNA can be isolated from samples using various techniques well known in the art (Nucleic Acids in the Environment Methods & Applications, J.T. Trevors, D.D. van Elsas, Springer Laboratory, 1995). Preferably, DNA obtained will be of large size and free of enzyme inhibitors or other contaminants. DNA can be isolated directly from an environmental sample (direct lysis), or cells may be harvested from the sample prior to DNA recovery (cell separation). Direct lysis procedures have several advantages over protocols based on cell separation. The direct lysis technique provides more DNA with a generally higher representation of the microbial community, however, it is sometimes smaller in size and more likely to contain enzyme inhibitors than DNA recovered using the cell separation technique. Very useful direct lysis techniques have been described which provide DNA of high molecular weight and high purity (Barns, 1994; Holben, 1994). If inhibitors are present, there are several protocols which utilize cell isolation which can be employed (Holben, 1994). Additionally, a fractionation technique, such as the bis-benzimide separation (cesium chloride isolation) described, can be used to enhance the purity of the DNA.

[0073] Isolation of total genomic DNA from extreme environmental samples varies depending on the source and quantity of material. Uncontaminated, good quality (>20 kbp) DNA is required for the construction of a representative library. A successful general DNA isolation protocol is the standard cetyl-trimethyl-ammonium-bromide (CTAB) precipitation technique. A biomass pellet is lysed and proteins digested by the nonspecific protease, proteinase K, in the presence of the detergent SDS. At elevated temperatures and high salt concentrations, CTAB forms insoluble complexes with denatured protein, polysaccharides and cell debris. Chloroform extractions are performed until the white interface containing the CTAB complexes is reduced substantially. The nucleic acids in the supernatant are precipitated with isopropanol and resuspended in TE buffer.

[0074] For cells which are recalcitrant to lysis, a combination of chemical and mechanical methods with cocktails of various cell-lysing enzymes may be employed. Isolated nucleic acid may then further be purified using small cesium gradients.

[0075] Gene libraries can be generated by inserting the DNA isolated or derived from a sample into a vector or a plasmid. Such vectors or plasmids are preferably those containing expression regulatory sequences, including promoters, enhancers and the like. Such polynucleotides can be part of a vector and/or a composition and still be isolated, in that such vector or composition is not part of its natural environment. Particularly preferred phage or plasmids and methods for introduction and packaging into them are described herein.

[0076] The following outlines a general procedure for producing libraries from both culturable and non-culturable organisms: obtain Biomass DNA Isolation (various methods), shear DNA (for example, with a 25 gauge needle), blunt DNA, methylate DNA, ligate to linkers, cut back linkers, size fractionate (for example, use a Sucrose Gradient), ligate to lambda expression vector, package (*in vitro* lambda packaging extract), plate on *E. coli* host and amplify

[0077] As detailed in Figure 1, cloning DNA fragments prepared by random cleavage of the target DNA generates a representative library. DNA dissolved in TE buffer is vigorously passed through a 25 gauge double-hubbed needle until the sheared fragments are in the desired size range. The DNA ends are "polished" or blunted with Mung Bean Nuclease, and EcoRI restriction sites in the target DNA are protected with EcoRI Methylase. EcoRI linkers (GGAATTCC) are ligated to the blunted/protected DNA using a very high molar ratio of linkers to target DNA. This lowers the probability of two DNA molecules ligating together to create a chimeric clone. The linkers are cut back with EcoRI restriction endonuclease and the DNA is size fractionated. The removal of sub-optimal DNA fragments and the small linkers is critical because ligation to the vector will result in recombinant molecules that are unpackageable, or the construction of a library containing only linkers as inserts.

Sucrose gradient fractionation is used since it is extremely easy, rapid and reliable. Although the sucrose gradients do not provide the resolution of agarose gel isolations, they do produce DNA that is relatively free of inhibiting contaminants. The prepared target DNA is ligated to the lambda vector, packaged using *in vitro* packaging extracts and grown on the appropriate *E. coli*.

[0078] As representative examples of expression vectors which may be used there may be mentioned viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral DNA (*e.g.* vaccinia, adenovirus, fowl pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as bacillus, aspergillus, yeast, *etc.*) Thus, for example, the DNA may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, (ZAP vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or other vector may be used as long as they are replicable and viable in the host.

[0079] Another type of vector for use in the present invention contains an f-factor origin replication. The f-factor (or fertility factor) in *E. coli* is a plasmid which effects high frequency transfer of itself during conjugation and less frequent transfer of the bacterial chromosome itself. A particularly preferred embodiment is to use cloning vectors, referred to as "fosmids" or bacterial artificial chromosome (BAC) vectors. These are derived from *E. coli* f-factor which is able to stably integrate large segments of genomic DNA. When integrated with DNA from a mixed uncultured environmental sample, this makes it possible to achieve large genomic fragments in the form of a stable "environmental DNA library."

[0080] The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct RNA synthesis. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers.

[0081] In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

[0082] Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), (-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium.

[0083] The cloning strategy permits expression via both vector driven and endogenous promoters; vector promotion may be important with expression of genes whose endogenous promoter will not function in *E. coli*.

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[0084] The DNA derived from a microorganism(s) may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

[0085] The DNA selected and isolated as hereinabove described is introduced into a suitable host to prepare a library which is screened for the desired enzyme activity. The selected DNA is preferably already in a vector which includes appropriate control sequences whereby selected DNA which encodes for an enzyme may be expressed, for detection of the desired activity. The host cell is a prokaryotic cell, such as a bacterial cell. Particularly preferred host cells are *E.coli*. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)). The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

[0086] Host cells are genetically engineered (transduced or transformed or transfected) with the vectors. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[0087] It is also contemplated that expression libraries generated can be phage display or cell surface display libraries. Numerous techniques are published in the art for generating such libraries.

[0088] After the expression libraries have been generated one can include the additional step of "biopanning" such libraries prior to screening by cell sorting. The "biopanning" procedure refers to a process for identifying clones having a specified

biological activity by screening for sequence homology in a library of clones prepared by (i) selectively isolating target DNA, from DNA derived from at least one microorganism, by use of at least one probe DNA comprising at least a portion of a DNA sequence encoding an biological having the specified biological activity; and (ii) optionally transforming a host with isolated target DNA to produce a library of clones which are screened for the specified biological activity.

[0089] The probe DNA used for selectively isolating the target DNA of interest from the DNA derived from at least one microorganism can be a full-length coding region sequence or a partial coding region sequence of DNA for an enzyme of known activity. The original DNA library can be preferably probed using mixtures of probes comprising at least a portion of the DNA sequence encoding an enzyme having the specified enzyme activity. These probes or probe libraries are preferably single-stranded and the microbial DNA which is probed has preferably been converted into single-stranded form. The probes that are particularly suitable are those derived from DNA encoding enzymes having an activity similar or identical to the specified enzyme activity which is to be screened.

[0090] The probe DNA should be at least about 10 bases and preferably at least 15 bases. In one embodiment, the entire coding region may be employed as a probe. Conditions for the hybridization in which target DNA is selectively isolated by the use of at least one DNA probe will be designed to provide a hybridization stringency of at least about 50% sequence identity, more particularly a stringency providing for a sequence identity of at least about 70%.

[0091] In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (e.g., GC v. AT content), and nucleic acid type (e.g., RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting

hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

[0092] An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

[0093] Hybridization techniques for probing a microbial DNA library to isolate target DNA of potential interest are well known in the art and any of those which are described in the literature are suitable for use herein, particularly those which use a solid phase-bound, directly or indirectly bound, probe DNA for ease in separation from the remainder of the DNA derived from the microorganisms.

[0094] Preferably the probe DNA is "labeled" with one partner of a specific binding pair (*i.e.* a ligand) and the other partner of the pair is bound to a solid matrix to provide ease of separation of target from its source. The ligand and specific binding partner can be selected from, in either orientation, the following: (1) an antigen or hapten and an antibody or specific binding fragment thereof; (2) biotin or iminobiotin and avidin or streptavidin; (3) a sugar and a lectin specific therefor; (4) an enzyme and an inhibitor therefor; (5) an apoenzyme and cofactor; (6) complementary homopolymeric oligonucleotides; and (7) a hormone and a receptor therefor. The solid phase is preferably selected from: (1) a glass or polymeric surface; (2) a packed column of polymeric beads; and (3) magnetic or paramagnetic particles.

[0095] Further, it is optional but desirable to perform an amplification of the target DNA that has been isolated. In this embodiment the target DNA is separated from the probe DNA after isolation. It is then amplified before being used to transform hosts. The double stranded DNA selected to include as at least a portion thereof a predetermined DNA sequence can be rendered single stranded, subjected to amplification and reannealed to provide amplified numbers of selected double stranded DNA. Numerous amplification methodologies are now well known in the art.

[0096] The selected DNA is then used for preparing a library for screening by transforming a suitable organism. Hosts, particularly those specifically identified herein as preferred, are transformed by artificial introduction of the vectors containing the target DNA by inoculation under conditions conducive for such transformation.

[0097] The resultant libraries of transformed clones are then screened for clones which display activity for the enzyme of interest.

[0098] Having prepared a multiplicity of clones from DNA selectively isolated from an organism, such clones are screened for a specific enzyme activity and to identify the clones having the specified enzyme characteristics.

[0099] The screening for enzyme activity may be effected on individual expression clones or may be initially effected on a mixture of expression clones to ascertain whether or not the mixture has one or more specified enzyme activities. If the mixture has a specified enzyme activity, then the individual clones may be rescreened utilizing a FACS machine for such enzyme activity or for a more specific activity. Alternatively, encapsulation techniques such as gel microdroplets, may be employed to localize multiple clones in one location to be screened on a FACS machine for positive expressing clones within the group of clones which can then be broken out into individual clones to be screened again on a FACS machine to identify positive individual clones. Thus, for example, if a clone mixture has hydrolase

activity, then the individual clones may be recovered and screened utilizing a FACS machine to determine which of such clones has hydrolase activity. As used herein, "small insert library" means a gene library containing clones with random small size nucleic acid inserts of up to approximately 5000 base pairs. As used herein, "large insert library" means a gene library containing clones with random large size nucleic acid inserts of approximately 5000 up to several hundred thousand base pairs or greater.

[0100] As described with respect to one of the above aspects, the invention provides a process for enzyme activity screening of clones containing selected DNA derived from a microorganism which process includes: screening a library for specified enzyme activity, said library including a plurality of clones, said clones having been prepared by recovering from genomic DNA of a microorganism selected DNA, which DNA is selected by hybridization to at least one DNA sequence which is all or a portion of a DNA sequence encoding an enzyme having the specified activity; and transforming a host with the selected DNA to produce clones which are screened for the specified enzyme activity.

[0101] In one embodiment, a DNA library derived from a microorganism is subjected to a selection procedure to select therefrom DNA which hybridizes to one or more probe DNA sequences which is all or a portion of a DNA sequence encoding an enzyme having the specified enzyme activity by:

- (a) rendering the double-stranded genomic DNA population into a single-stranded DNA population;
- (b) contacting the single-stranded DNA population of (a) with the DNA probe bound to a ligand under conditions permissive of hybridization so as to produce a double-stranded complex of probe and members of the genomic DNA population which hybridize thereto; (c) contacting the double-stranded complex of (b) with a solid phase specific binding partner for said ligand so as to produce a solid phase complex;

- (d) separating the solid phase complex from the single-stranded DNA population of (b);
- (e) releasing from the probe the members of the genomic population which had bound to the solid phase bound probe;
- (f) forming double-stranded DNA from the members of the genomic population of (e);
- (g) introducing the double-stranded DNA of (f) into a suitable host to form a library containing a plurality of clones containing the selected DNA; and
- (h) screening the library for the specified enzyme activity.

[0102] In another aspect, the process includes a preselection to recover DNA including signal or secretion sequences. In this manner it is possible to select from the genomic DNA population by hybridization as hereinabove described only DNA which includes a signal or secretion sequence. The following paragraphs describe the protocol for this embodiment of the invention, the nature and function of secretion signal sequences in general and a specific exemplary application of such sequences to an assay or selection process.

[0103] A particularly preferred embodiment of this aspect further comprises, after (a) but before (b) above, the steps of:

- (a *i*). contacting the single-stranded DNA population of (a) with a ligand-bound oligonucleotide probe that is complementary to a secretion signal sequence unique to a given class of proteins under conditions permissive of hybridization to form a double-stranded complex;
- (a *ii*). contacting the double-stranded complex of (a *i*) with a solid phase specific binding partner for said ligand so as to produce a solid phase complex;
- (a *iii*) separating the solid phase complex from the single-stranded DNA population of (a);
- (a *iv*) releasing the members of the genomic population which had bound to said solid phase bound probe; and (a *v*) separating the solid phase bound probe from the members of the genomic population which had bound thereto.

[0104] The DNA which has been selected and isolated to include a signal sequence is then subjected to the selection procedure hereinabove described to select and isolate therefrom DNA which binds to one or more probe DNA sequences derived from DNA encoding an enzyme(s) having the specified enzyme activity.

[0105] This procedure is described and exemplified in U.S. Serial No. 08/692,002, filed August 2, 1996, ^{now U.S. Patent No. 6,054,267} incorporated herein by reference.

[0106] In-vivo biopanning may be performed utilizing a FACS-based machine. Complex gene libraries are constructed with vectors which contain elements which stabilize transcribed RNA. For example, the inclusion of sequences which result in secondary structures such as hairpins which are designed to flank the transcribed regions of the RNA would serve to enhance their stability, thus increasing their half life within the cell. The probe molecules used in the biopanning process consist of oligonucleotides labeled with reporter molecules that only fluoresce upon binding of the probe to a target molecule. These probes are introduced into the recombinant cells from the library using one of several transformation methods. The probe molecules bind to the transcribed target mRNA resulting in DNA/RNA heteroduplex molecules. Binding of the probe to a target will yield a fluorescent signal which is detected and sorted by the FACS machine during the screening process.

[0107] Further, it is possible to combine all the above embodiments such that a normalization step is performed prior to generation of the expression library, the expression library is then generated, the expression library so generated is then biopanned, and the biopanned expression library is then screened using a high throughput cell sorting and screening instrument. Thus there are a variety of options: *i.e.* (i) one can just generate the library and then screen it; (ii) normalize the target DNA, generate the expression library and screen it; (iii) normalize, generate the library, biopan and screen; or (iv) generate, biopan and screen the library.

[0108] The library may, for example, be screened for a specified enzyme activity. For example, the enzyme activity screened for may be one or more of the six IUB classes; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. The recombinant enzymes which are determined to be positive for one or more of the IUB classes may then be rescreened for a more specific enzyme activity.

[0109] Alternatively, the library may be screened for a more specialized enzyme activity. For example, instead of generically screening for hydrolase activity, the library may be screened for a more specialized activity, *i.e.* the type of bond on which the hydrolase acts. Thus, for example, the library may be screened to ascertain those hydrolases which act on one or more specified chemical functionalities, such as: (a) amide (peptide bonds), *i.e.* proteases; (b) ester bonds, *i.e.* esterases and lipases; (c) acetals, *i.e.*, glycosidases *etc.*

[0110] The clones which are identified as having the specified enzyme activity may then be sequenced to identify the DNA sequence encoding an enzyme having the specified activity. Thus, in accordance with the present invention it is possible to isolate and identify: (i) DNA encoding an enzyme having a specified enzyme activity, (ii) enzymes having such activity (including the amino acid sequence thereof) and (iii) produce recombinant enzymes having such activity.

[0111] The present invention may be employed for example, to identify new enzymes having, for example, the following activities which may be employed for the following uses:

[0112] **Lipase/Esterase**

[0113] Enantioselective hydrolysis of esters (lipids)/ thioesters, resolution of racemic mixtures, synthesis of optically active acids or alcohols from *meso*-diesters, selective syntheses, regiospecific hydrolysis of carbohydrate esters, selective hydrolysis of cyclic secondary alcohols, synthesis of optically active esters, lactones, acids, alcohols, transesterification of activated/nonactivated esters, interesterification,

optically active lactones from hydroxyesters, egio- and enantioselective ring opening of anhydrides, detergents, fat/oil conversion and cheese ripening.

[0114] Protease

[0115] Ester/amide synthesis, peptide synthesis, resolution of racemic mixtures of amino acid esters, synthesis of non-natural amino acids and detergents/protein hydrolysis.

[0116] Glycosidase/Glycosyl transferase

[0117] Sugar/polymer synthesis, cleavage of glycosidic linkages to form mono, di- and oligosaccharides, synthesis of complex oligosaccharides, glycoside synthesis using UDP-galactosyl transferase, transglycosylation of disaccharides, glycosyl fluorides, aryl galactosides, glycosyl transfer in oligosaccharide synthesis, diastereoselective cleavage of α -glucosylsulfoxides, asymmetric glycosylations, food processing and paper processing.

[0118] Phosphatase/Kinase

[0119] Synthesis/hydrolysis of phosphate esters, regio- and enantioselective phosphorylation, introduction of phosphate esters, synthesize phospholipid precursors, controlled polynucleotide synthesis, activate biological molecule, selective phosphate bond formation without protecting groups.

[0120] Mono/Dioxygenase

[0121] Direct oxyfunctionalization of unactivated organic substrates, hydroxylation of alkane, aromatics, steroids, epoxidation of alkenes, enantioselective sulfoxidation, regio- and stereoselective Bayer-Villiger oxidations.

[0122] Haloperoxidase

[0123] Oxidative addition of halide ion to nucleophilic sites, addition of hypohalous acids to olefinic bonds, ring cleavage of cyclopropanes, activated

aromatic substrates converted to *ortho* and *para* derivatives
1.3 diketones converted to 2-halo-derivatives, heteroatom oxidation of sulfur and nitrogen containing substrates, oxidation of enol acetates, alkynes and activated aromatic rings

[0124] Lignin peroxidase/Diarylpropane peroxidase

[0125] Oxidative cleavage of C-C bonds, oxidation of benzylic alcohols to aldehydes, hydroxylation of benzylic carbons, phenol dimerization, hydroxylation of double bonds to form diols, cleavage of lignin aldehydes.

[0126] Epoxide hydrolase

[0127] Synthesis of enantiomerically pure bioactive compounds, regio- and enantioselective hydrolysis of epoxide, aromatic and olefinic epoxidation by monooxygenases to form epoxides, resolution of racemic epoxides, hydrolysis of steroid epoxides.

[0128] Nitrile hydratase/nitrilase

[0129] Hydrolysis of aliphatic nitriles to carboxamides, hydrolysis of aromatic, heterocyclic, unsaturated aliphatic nitriles to corresponding acids, hydrolysis of acrylonitrile, production of aromatic and carboxamides, carboxylic acids (nicotinamide, picolinamide, isonicotinamide), regioselective hydrolysis of acrylic dinitrile, amino acids from hydroxynitriles.

[0130] Transaminase

[0131] Transfer of amino groups into oxo-acids.

[0132] Amidase/Acylase

[0133] Hydrolysis of amides, amidines, and other C-N bonds, non-natural amino acid resolution and synthesis.

[0134] As indicated, the present invention also offers the ability to screen for other types of bioactivities. For instance, the ability to select and combine desired components from a library of polyketides and postpolyketide biosynthesis genes for

generation of novel polyketides for study is appealing. The method(s) of the present invention make it possible to and facilitate the cloning of novel polyketide synthases, since one can generate gene banks with clones containing large inserts (especially when using vectors which can accept large inserts, such as the f-factor based vectors), which facilitates cloning of gene clusters.

[0135] Preferably, the gene cluster or pathway DNA is ligated into a vector, particularly wherein a vector further comprises expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. As previously indicated, this f-factor of *E. coli* is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. Other examples of vectors include cosmids, bacterial artificial chromosome vectors, and P1 vectors.

[0136] Lambda vectors can also accommodate relatively large DNA molecules, have high cloning and packaging efficiencies and are easy to handle and store compared to plasmid vectors. (-ZAP vectors (Stratagene Cloning Systems, Inc.) have a convenient subcloning feature that allows clones in the vector to be excised with helper phage into the pBluescript phagemid, eliminating the time involved in subcloning. The cloning site in these vectors lies downstream of the *lac* promoter. This feature allows expression of genes whose endogenous promoter does not function in *E. coli*.

[0137] The following describes the total number of assays required to test an entire library:

[0138] The two main factors which govern the total number of clones that can be pooled and simultaneously screened are (i) the level of gene expression and (ii) enzyme assay sensitivity. As estimate of the level of gene expression is that each *E. coli* cell infected with lambda will produce 10^3 copies of the gene product from the insert. FACS instruments are sufficiently sensitive to detect about 500 to 1000 Fluorescein molecules.

[0139] In order to assess the total number of clones to be tested (*e.g.*, the number of genome equivalents) a statistical analysis was performed. Assuming that mechanical shearing and gradient purification results in a normal distribution of DNA fragment sizes with a mean of 4.5 kbp and variance of 1 kbp, the fraction represented of all possible 1 kbp sequences in a 1.8 Mbp genome is plotted in Figure 3 as a function of increasing genome equivalents.

[0140] Based on these results, approximately 2,000 clones (5 genome equivalents) must be screened to achieve a ~90% probability of obtaining a particular gene. This represents the point of maximal efficiency for library throughput. Assuming that a complex environmental library contains about 1000 different organisms, at least 2,000,000 clones have to be screened to achieve a >90% probability of obtaining a particular gene. This number rises dramatically assuming that the organisms differ vastly in abundance in natural populations.

[0141] Substrate can be administered to the cells before or during the process of the cell sorting analysis. In either case a solution of the substrate is made up and the cells are contacted therewith. When done prior to the cell sorting analysis this can be by making a solution which can be administered to the cells while in culture plates or other containers. The concentration ranges for substrate solutions will vary according to the substrate utilized. Commercially available substrates will generally contain instructions on concentration ranges to be utilized for, for instance, cell staining purposes. These ranges may be employed in the determination of an optimal concentration or concentration range to be utilized in the present invention. The

substrate solution is maintained in contact with the cells for a period of time and at an appropriate temperature necessary for the substrate to permeablize the cell membrane. Again, this will vary with substrate. Instruments which deliver reagents in stream such as by poppet valves which seal openings in the flow path until activated to permit introduction of reagents (*e.g.* substrate) into the flow path in which the cells are moving through the analyzer can be employed for substrate delivery.

[0142] The substrate is one which is able to enter the cell and maintain its presence within the cell for a period sufficient for analysis to occur. It has generally been observed that introduction of the substrate into the cell across the cell membrane occurs without difficulty. It is also preferable that once the substrate is in the cell it not "leak" back out before reacting with the biomolecule being sought to an extent sufficient to product a detectable response. Retention of the substrate in the cell can be enhanced by a variety of techniques. In one, the substrate compound is structurally modified by addition of a hydrophobic tail. In another certain preferred solvents, such as DMSO or glycerol, can be administered to coat the exterior of the cell. Also the substrate can be administered to the cells at reduced temperature which has been observed to retard leakage of the substrate from the cell's interior.

[0143] A broad spectrum of substrates can be used which are chosen based on the type of bioactivity sought. In addition where the bioactivity being sought is in the same class as that of other biomolecules for which a number have known substrates, the bioactivity can be examined using a cocktail of the known substrates for the related biomolecules which are already known. For example, substrates are known for approximately 20 commercially available esterases and the combination of these known substrates can provide detectable, if not optimal, signal production. Substrates are also known and available for glycosidases, proteases, phosphatases, and monooxygenases.

[0144] The substrate interacts with the target biomolecule so as to produce a detectable response. Such responses can include chromogenic or fluorogenic

2000年12月20日，中国科学院南京地质古生物研究所的科研人员，在南京六合县瓜埠镇瓜埠村，发现了一处新的古生物化石产地。

[0146] In comparison to colorimetric assays, fluorescent based assays are very sensitive, which is a major criteria for single cell assays. There are two main factors which govern the screening of a recombinant enzyme in a single cell: i) the level of gene expression, and ii) enzyme assay sensitivity. To estimate the level of gene expression one can determine how many copies of the gene product will be produced by the host cell given the vector. For instance, one can assume that each *E. coli* cell infected with pBluescript phagemid (Stratagene Cloning Systems, Inc.) will produce $\sim 10^3$ copies of the gene product from the insert. The FACS instruments are capable of detecting about 500 to 1,000 fluorescein molecules per cell. Assuming that one enzyme turns over at least one fluorescein based substrate molecule, one cell will display enough fluorescence to be detected by the optics of a fluorescence-activated cell sorter (FACS).

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(FDG) as a substrate for β -galactosidase, which releases fluorescein, a product that can be detected by a fluorescence-activated cell sorter (FACS) upon hydrolysis (Nolan *et al.*, 1991). A problem with the use of FDG is that if the assay is performed at room temperature, the fluorescence leaks out of the positively stained cells. A similar problem was encountered in other studies of β -galactosidase measurements in mammalian cells and yeast with FDG as well as other substrates (Nolan *et al.*, 1988; Wittrup *et al.*, 1988). Performing the reaction at 0°C appreciably decreased the extent of this leakage of fluorescence (Nolan *et al.*, 1988). However this low temperature is not adaptable for screening for, for instance, high temperature β -galactosidases. Other fluorogenic substrates have been developed, such as 5-dodecanoylamino fluorescein di-(-D-galactopyranoside (C₁₂-FDG) (Molecular Probes) which differs from FDG in that it is a lipophilic fluorescein derivative that can easily cross most cell membranes under physiological culture conditions. The green fluorescent enzymatic hydrolysis product is retained for hours to days in the membrane of those cells that actively express the *lacZ* reporter gene. In animal cells C₁₂-FDG was a much better substrate, giving a signal which was 100 times higher than the one obtained with FDG (Plovins *et al.*, 1994). However in Gram negative bacteria like *E. coli*, the outer membrane functions as a barrier for the lipophilic molecule C₁₂-FDG and it only passes through this barrier if the cells are dead or damaged (Plovins *et al.*). The fact that C₁₂ retains FDG substrate inside the cells indicates that the addition of unpolarized tails may be used for retaining substrate inside the cells with respect to other enzyme substrates.

[0148] The abovementioned β -galactosidase assays may be employed to screen single *E. coli* cells, expressing recombinant β -D-galactosidase isolated from a hyperthermophilic archaeon such as *Sulfolobus solfataricus*, on a fluorescent microscope. Cells are cultivated overnight, centrifuged and washed in deionized water and stained with FDG. To increase enzyme activity, cells are heated to 70°C for 30 minutes and examined with a fluorescence phase contrast microscope. *E. coli* cell suspensions of the β -galactosidase expressing clone stained with C₁₂-FDG show a very bright fluorescence inside single cells (Fig 8).

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[illegible]

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overexpression. Alternatively, expressing clones can be "bulk sorted" into single tubes and the plasmid inserts recovered as amplified products, which are then subcloned and transformed into suitable vector-hosts systems for rescreening.

[0156] Encapsulation techniques may be employed to localize signal, even in cases where cells are no longer viable. Gel microdrops (GMDs) are small (25 to 50um in diameter) particles made with a biocompatible matrix. In cases of viable cells, these microdrops serve as miniaturized petri dishes because cell progeny are retained next to each other, allowing isolation of cells based on clonal growth. The basic method has a significant degree of automation and high throughput; after the colony size signal boundaries are established, about 10^6 GMDs per hour can be automatically processed. Cells are encapsulated together with substrates and particles containing a positive clones are sorted. Fluorescent substrate labeled glass beads can also be loaded inside the GMDs. In cases of non-viable cells, GMDs can be employed to ensure localization of signal.

[0157] After viable or non-viable cells, each containing a different expression clone from the gene library are screened on a FACS machine, and positive clones are recovered, DNA is isolated from positive clones. The DNA can then be amplified either *in vivo* or *in vitro* by utilizing any of the various amplification techniques known in the art. *In vivo* amplification would include transformation of the clone(s) or subclone(s) of the clones into a viable host, followed by growth of the host. *In vitro* amplification can be performed using techniques such as the polymerase chain reaction.

[0158] Clones found to have the bioactivity for which the screen was performed can also be subjected to directed mutagenesis to develop new bioactivities with desired properties or to develop modified bioactivities with particularly desired properties that are absent or less pronounced in the wild-type enzyme, such as stability to heat or organic solvents. Any of the known techniques for directed mutagenesis are applicable to the invention. For example, particularly preferred

mutagenesis techniques for use in accordance with the invention include those described below.

[0159] The term "error-prone PCR" refers to a process for performing PCR under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. Leung, D.W., *et al.*, Technique, 1:11-15 (1989) and Caldwell, R.C. & Joyce G.F., PCR Methods Applic., 2:28-33 (1992).

[0160] The term "oligonucleotide directed mutagenesis" refers to a process which allows for the generation of site-specific mutations in any cloned DNA segment of interest. Reidhaar-Olson, J.F. & Sauer, R.T., *et al.*, Science, 241:53-57 (1988).

[0161] The term "assembly PCR" refers to a process which involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of different PCR reactions occur in parallel in the same vial, with the products of one reaction priming the products of another reaction.

[0162] The term "sexual PCR mutagenesis" (also known as "DNA shuffling") refers to forced homologous recombination between DNA molecules of different but highly related DNA sequence *in vitro*, caused by random fragmentation of the DNA molecule based on sequence homology, followed by fixation of the crossover by primer extension in a PCR reaction. Stemmer, W.P., PNAS, USA, 91:10747-10751 (1994).

[0163] The term "*in vivo* mutagenesis" refers to a process of generating random mutations in any cloned DNA of interest which involves the propagation of the DNA in a strain of *E. coli* that carries mutations in one or more of the DNA repair pathways. These "mutator" strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will eventually generate random mutations within the DNA.

[0164] The term "cassette mutagenesis" refers to any process for replacing a small region of a double stranded DNA molecule with a synthetic oligonucleotide "cassette" that differs from the native sequence. The oligonucleotide often contains completely and/or partially randomized native sequence.

[0165] The term "recursive ensemble mutagenesis" refers to an algorithm for protein engineering (protein mutagenesis) developed to produce diverse populations of phenotypically related mutants whose members differ in amino acid sequence. This method uses a feedback mechanism to control successive rounds of combinatorial cassette mutagenesis. Arkin, A.P. and Youvan, D.C., PNAS, USA, 89:7811-7815 (1992).

[0166] The term "exponential ensemble mutagenesis" refers to a process for generating combinatorial libraries with a high percentage of unique and functional mutants, wherein small groups of residues are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins, Delegrave, S. and Youvan, D.C., Biotechnology Research, 11:1548-1552 (1993); and random and site-directed mutagenesis, Arnold, F.H., Current Opinion in Biotechnology, 4:450-455 (1993).

[0167] All of the references mentioned above are hereby incorporated by reference in their entirety. Each of these techniques is described in detail in the references mentioned.

[0168] DNA can be mutagenized, or "evolved", utilizing any one or more of these techniques, and rescreened on the FACS machine to identify more desirable clones. "Fluorescence screening" as utilized herein means screening for any activity of interest utilizing any fluorescent analyzer that detects fluorescence. Internal control reference genes which either express fluorescing molecules, such as those encoding green fluorescent protein, or encode proteins that can turnover fluorescing molecules,

such as beta-galactosidase, can be utilized. These internal controls should optimally fluoresce at a wavelength which is different from the wavelength at which the molecule used to detect the evolved molecule(s) emits. DNA is evolved, recombined in a vector which co-expresses these proteins or molecules, transformed into an appropriate host organism, and rescreened utilizing the FACS machine to identify more desirable clones.

[0169] An important aspect of the invention is that cells are being analyzed individually. However other embodiments are contemplated which involve pooling of cells and multiple passage screen. This provides for a tiered analysis of biological activity from more general categories of activity, *i.e.* categories of enzymes, to specific activities of principle interest such as enzymes of that category which are specific to particular substrate molecules.

[0170] Members of these libraries can be encapsulated in gel microdroplets, exposed to substrates of interest, such as transition state analogs, and screened based on binding via FACS sorting for activities of interest.

[0171] It is anticipated with the present invention that one could employ mixtures of substrates to simultaneously detect multiple activities of interest simultaneously or sequentially. FACS instruments can detect molecules that fluoresce at different wavelengths, hence substrates which fluoresce at different wavelengths and indicate different activities can be employed.

[0172] The fluorescence activated cell sorting screening method of the present invention allows one to assay several million clones per hour for a desired bioactivity. This technique provides an extremely high throughput screening process necessary for the screening of extreme biodiverse environmental libraries.

[0173] In a preferred embodiment, the present invention provides a novel method for screening for activities, defined as "agents" herein, which affect the action of

transducing proteins, such as, for example, G-proteins. In the present invention, cells containing functional transducing proteins (such as membrane bound G-proteins), defined herein as "target cells" or "target(s)", are co-encapsulated with potential agent molecules and screened for affects agent molecules may have on their actions. Potential agent molecules are originally derived from a gene library generated from environmental or other samples, as described herein.

[0174] In particular, agents are molecules encoded by a pathway or gene cluster, or molecules generated by the expression of said pathways or clusters. Cells containing nucleic acid expressing the agent, or cells containing nucleic acid expressing activities which act within the cell to yield agent molecules can be utilized for screening. Alternatively, agent molecules can be expressed or generated prior to screening, and subsequently utilized. Cells expressing agent molecules, or agent molecules are coencapsulated, and screened utilizing various methods, such as those described herein.

[0175] Agent molecules can exist in or be introduced into the encapsulation particle by various means. Cells expressing genes encoding proteins which act to generate agent molecules (small molecules, for example) can be introduced into encapsulation particles using, for instance, Examples provided herein. Said cells can be prokaryotic or eukaryotic cells. Prokaryotic cells can be bacteria, such as *E.coli*. As previously indicated, genes can alternatively be expressed outside the encapsulation particle, the expression product or molecules generated via action of expressed products (such as small molecules or agent molecules) can be purified from the host, and said agents may be introduced into the encapsulation particle with the functional transducing protein(s), also using the methods described in the Examples below.

[0176] Encapsulation can be in beads, high temperature agaroses, gel microdroplets, cells, such as ghost red blood cells or macrophages, liposomes, or any other means of encapsulating and localizing molecules.

[0177] For example, methods of preparing liposomes have been described (*i.e.*, U.S. Patent No.'s 5,653,996, 5,393,530 and 5,651,981), as well as the use of liposomes to encapsulate a variety of molecules U.S. Patent No.'s 5,595,756, 5,605,703, 5,627,159, 5,652,225, 5,567,433, 4,235,871, 5,227,170). Entrapment of proteins, viruses, bacteria and DNA in erythrocytes during endocytosis has been described, as well (Journal of Applied Biochemistry 4, 418-435 (1982)). Erythrocytes employed as carriers in vitro or in vivo for substances entrapped during hypo-osmotic lysis or dielectric breakdown of the membrane have also been described (reviewed in Ihler, G. M. (1983) J. Pharm. Ther). These techniques are useful in the present invention to encapsulate samples for screening.

[0178] "Microenvironment", as used herein, is any molecular structure which provides an appropriate environment for facilitating the interactions necessary for the method of the invention. An environment suitable for facilitating molecular interactions include, for example, liposomes. Liposomes can be prepared from a variety of lipids including phospholipids, glycolipids, steroids, long-chain alkyl esters; *e.g.*, alkyl phosphates, fatty acid esters; *e.g.*, lecithin, fatty amines and the like. A mixture of fatty material may be employed such a combination of neutral steroid, a charge amphiphile and a phospholipid. Illustrative examples of phospholipids include lecithin, sphingomyelin and dipalmitoylphosphatidylcholine. Representative steroids include cholesterol, cholestanol and lanosterol. Representative charged amphiphilic compounds generally contain from 12-30 carbon atoms. Mono- or dialkyl phosphate esters, or alkyl amines; *e.g.*, dicetyl phosphate, stearyl amine, hexadecyl amine, dilauryl phosphate, and the like.

[0179] In addition, agents which potentially enhance or inhibit ligand/receptor interactions may be screened and identified. Thus, the present invention thus provides a method to screen recombinants producing drugs which block or enhance interactions of molecules, such as protein-protein interactions. When screening for compounds which affect G-protein interactions, host cells expressing recombinant

clones to be screened are co-encapsulated with membrane bound G-proteins and ligands. Compounds (such as small molecules) diffuse out of host cells, and enhancement or inhibition of G-protein interactions can be evaluated via a variety of methods. Any screening method which allows one to detect an increase or decrease in activity or presence of an intracellular compound or molecule, including nucleic acids and proteins, which results from enhancement or inhibition of ligand/receptor interactions, transducers, such as G-protein interactions, or cascade events occurring inside a cell are useful in the present invention.

[0180] For example, the adenylyl cyclase method described above can be utilized in the present invention. Other assays which detect effects, or changes, modulated by effectors are useful in the present invention. The change, or signal, must be detectable against the background, or basal activity of the effector in the absence of the potential small molecule or drug. The signal may be a change in the growth rate of the cells, or other phenotypic changes, such as a color change or luminescence. Production of functional gene products may be impacted by the effect, as well. For example, the production of a functional gene product which is normally regulated by downstream or direct effects created by the transducer or effector can be altered and detected. Said functional genes may include reporter molecules, such as green fluorescent protein, or red fluorescent protein (Biosci Biotechnol Biochem 1995 Oct; 59(10):1817-1824), or other detectable molecules. These "functional genes" are used as marker genes. "Marker genes" are engineered into the host cell where desired. Modifications to their expression levels causes a phenotypic or other change which is screenable or selectable. If the change is selectable, a phenotypic change creates a difference in the growth or survival rate between cells which express the marker gene and those which do not, or a detectable modification in expression levels of reporter molecules within or around cells. If the change is screenable, the phenotype change creates a difference in some detectable characteristic of the cells, by which the cells which express the marker may be distinguished from those which do not. Selection is preferable to screening.

[0181] Rapid assays which measure direct readouts of transcriptional activity are useful in the present invention. For example, placing the bacterial gene encoding lacZ under the control of the FUS1 promoter, activation of the yeast pheromone response pathway can be detected in less than an hour by monitoring the ability of permeabilized yeast to produce color from a chromogenic substrate. Activation of other response pathways may be assayed via similar strategies. Genes encoding detectable molecules, or which create a detectable signal via modification of another molecules, can be utilized to analyze activation or suppression of a response.

[0182] The use of fluorescent proteins and/or fluorescent groups and quenching groups in close proximity to one another to assay the presence of enzymes or nucleic acid sequences has been reported (WO 97/28261 and WO 95/13399). In the first of these reactions, fluorescent proteins having the proper emission and excitation spectra are put in physically close proximity to exhibit fluorescence energy transfer. Substrates for enzyme activities are placed between the two proteins, such that cleavage of the substrate by the presence of the enzymatic activity separates the proteins enough to change the emission spectra. Another group utilizes a fluorescent protein and a quencher molecule in close proximity to exhibit "collisional quenching" properties whereby the fluorescence of the fluorescent protein is diminished simply via the proximity of the quenching group. Probe nucleic acid sequences are engineered between the two groups, and a hybridization event between the probe sequence and a target in a sample separates the protein from the quencher enough to yield a fluorescent signal. Still another group has reported a combination of the above strategies, engineering a molecule which utilizes an enzyme substrate flanked by a fluorescent protein on one end and a quencher on the other (EP 0 428 000). It is recognized that these types assays can be employed in the method of the present invention to detect modifications in nucleic acid production (transcriptional activation or repression) and/or enzyme or other protein production (translational modifications) which results from inhibition of or improved association of interacting molecules, such as ligands and receptors, or which results from actions of bioactive compounds directly on transcription of particular molecules.

[0183] Fluorescent proteins encoded by genes which can be used to transform host cells and employed in a screen to identify compounds of interest are particularly useful in the present invention. Substrates are localized into the encapsulation means by a variety of methods, including but not limited to the method described herein in the Example below. Cells can also be engineered to contain genes encoding fluorescing molecules. For example, transcriptionally regulated genes can be linked to reporter molecule genes to allow expression (or lack of expression) of the reporter molecule to facilitate detection of the expression of the transcriptionally regulated gene. For example, if the ultimate effect of an agonist or antagonist interacting to enhance or inhibit the binding of a ligand to a receptor, or to enhance or inhibit the effects of any molecule in a pathway, is transcriptional activation or repression of a gene of interest the cell, it is useful to be able to link the activated gene to a reporter gene to facilitate detection of the expression.

[0184] Cells can be engineered in variety of ways to allow the assay of the effect of compounds on cellular "events". An "event", as utilized herein, means any cellular function which is modified or event which occurs in response to exposure of the cell, or components of the cell, to molecules expressed by, or ultimately yielded by the expression of, members of gene libraries derived from samples and generated according to the methods described herein. For example, cellular events which can be detected with commercially available products include changes in transmembrane pH (*i.e.*, BCECF pH indicator sold by BioRad Laboratories, Inc., Hercules, California), cell cycle events, such as cell proliferation, cytotoxicity and cell death (*i.e.*, propidium iodide, 5-bromo-2'-deoxy-uridine (BrdU), Annexin-V-FLUOS, and TUNEL (method) sold by Boehringer-Mannheim Research Biochemicals), or production of proteins, such as enzymes. In many instances, the cascade of events begun by membrane protein interactions with other molecules involves modifications, such as phosphorylation or dephosphorylation, of molecules within the cell. Molecules, such as fluorescent substrates, which facilitate detection of these events are useful in the present invention to screen libraries expressing activities of interest.

ELISA or colorimetric assays can also be adapted to single cell screening to be utilized to screen libraries according to the present invention.

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[0185] Probe nucleic acid sequences designed according to the method described above can also be utilized in the present invention to "enrich" a population for desirable clones. "Enrich", as utilized herein, means reducing the number and/or complexity of an original population of molecules. For example, probes are designed to identify specific polyketide sequences, and utilized to enrich for clones encoding polyketide pathways. Figure X depicts in-situ hybridization of encapsulated fosmid clones with specific probes of interest, in this case polyketide synthase gene probes. Fosmid libraries are generated in E.coli according to the methods described in the Example herein. Clones are encapsulated and grown to yield encapsulated clonal populations. Cells are lysed and neutralized, and exposed to the probe of interest. Hybridization yields a positive fluorescent signal which can be sorted on a fluorescent cell sorter. Positives can be further screened via expression, or activity, screening. Thus, this aspect of the present invention facilitates the reduction of the complexity of the original population to enrich for desirable pathway clones. These clones can be utilized for further downstream screening. For example, these clones can be expressed to yield backbone structures (defined herein), which can be decorated in metabolically rich hosts, and finally screened for an activity of interest. Alternatively, clones can be expressed to yield small molecules directly, which can be screened for an activity of interest. Further more, multiple probes can be designed and utilized to allow "multiplex" screening and/or enrichment. "Multiplex" screening and/or enrichment as used herein means that one is screening and/or enriching for more than desirable outcome, simultaneously.

[0186] Detectable molecules may be added as substrates to be utilized in screening assays, or genes encoding detectable molecules may be utilized in the method of the present invention.

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S. venezuelae, unlike many other strains, grows in liquid grown cultures. As the cultures reach the end of the exponential growth phase, secondary metabolites, including bioactive compounds, are produced. It is possible that the production of these bioactive compounds is related to the natural fluorescence of the organism.

[0190] In one aspect of the present invention, any *Streptomyces* or *Actinomyces* species that can be manipulated to produce single cells or fragmented mycelia is screened for a characteristic of interest. It is preferable to screen cells at the stage in their life cycle when they are producing small molecules for purposes of the present invention.

[0191] A fluorescence-based method for the selection of recombinant plasmids has been reported (BioTechniques 19:760-764, November 1995). *Escherichia coli* strains containing plasmids for the overexpression of the gene encoding uroporphyrinogen III methyltransferase accumulate fluorescent porphyrinoid

compounds, which, when illuminated with ultraviolet light, causes recombinant cells to fluoresce with a bright red color. Replacement or disruption of the gene with other DNA fragments results in the loss of enzymatic activity and nonfluorescent cells.

[0192] Uroporphyrinogen III methyltransferase is an enzyme that catalyzes the S-adenosyl-l-methionine (SAM) -dependent addition of two methyl groups to uroporphyrinogen III methyltransferase to yield dihydrosirohydro-chlorin necessary for the synthesis of siroheme, factor F430 and vitamin B12. The substrate for this enzyme, uroporphyrinogen III (derived from γ -aminolevulinic acid) is a ubiquitous compound found not only in these pathways, but also in the pathways for the synthesis of the other so-called "pigments of life", heme and chlorophyll. Dihydrosirohydrochlorin is oxidated in the cell to produce a fluorescent compound sirohydrochlorin (Factor II) or modified again by uroporphyrinogen III methyltransferase to produce trimethylpyrrocorphin, another fluorescent compound. These fluorescent compounds fluoresce with a bright red to red-orange color when illuminated with UV light (300nm).

[0193] Bacterial uroporphyrinogen III methylases have been purified from *E.coli* (1), *Pseudomonas* (2), *Bacillus* (3) and *Methanobacterium* (4). A *Bacillus stearothermophilus* uroporphyrinogen III methylase has been cloned sequenced and expressed in *E.coli* (Biosci Biotechnol Biochem 1995 Oct; 59(10):1817-1824).

[0194] In the method of the present invention, the fluorescing properties of this and other similar compounds can be utilized to screen for compounds of interest, as described previously, or are utilized to enrich for the presence of compounds of interest. Host cells expressing recombinant clones potentially encoding gene pathways are screened for fluorescing properties. Thus, cells producing fluorescent proteins or metabolites can be identified. Pathway clones expressed in *E.coli* or other host cells, can yield bioactive compounds or "backbone structures" to bioactive compounds (which can subsequently be "decorated" in other host cells, for example, in metabolically rich organisms). The "backbone structure" is the fundamental

structure that defines a particular class of small molecules. For example, a polyketide backbone will differ from that of a lactone, a glycoside or a peptide antibiotic. Within each class, variants are produced by the addition or subtraction of side groups or by rearrangement of ring structures ("decoration" or "decorated"). Ring structures present in aromatic bioactive compounds are known in some instance to yield a fluorescent signal, which can be utilized to distinguish these cells from the population. Certain of these structures can also provide absorbance characteristics which differ from the background absorbance of a non-recombinant host cell, and thus can allow one to distinguish these cells from the population, as well. Recombinant cells potentially producing bioactive compounds or "backbone" structures can be identified and separated from a population of cells, thus enriching the population for desirable cells. Thus, the method of the present invention also facilitates the discovery of novel aromatic compounds encoded by gene pathways, for example, encoded by polyketide genes, directly from environmental or other samples.

[0195] Compounds can also be generated via the modification of host porphyrin-like molecules by gene products derived from these samples. Thus, one can screen for recombinant clone gene products which modify a host porphyrin-like compound to make it fluoresce.

[0196] In yet another aspect of the present invention, cells expressing molecules of interest are sorted into 96-well or 384-well plates, specifically for further downstream manipulation and screening for recombinant clones. In this aspect of the present invention, the a fluorescence analyzer, such as a FACS machine is employed not to distinguish members of and evaluate populations or to screen as previously published, but to screen and recover positives in a manner that allows further screens to be performed on samples selected. For example, typical stains used for enumeration can affect cell viability, therefore these types of stains were not employed for screening and selecting for further downstream manipulation of cells, specifically for the purpose, for example, of recovering nucleic acid which encodes an activity of interest. In particular, cells containing recombinant clones can be

identified and sorted into multi-well plates for further downstream manipulation. There are various ways of screening for the presence of a recombinant clone in a cell. Genes encoding fluorescent proteins, such as green fluorescent protein (Biotechniques 19(4):650-655, 1995), or the gene encoding uroporphyrinogen III methyltransferase (BioTechniques 19:760-764, November 1995) can be utilized in the method of the present invention as reporters to allow detection of recombinant clones. Recombinant clones are sorted for further downstream screening for an activity of interest. Screening may be for an enzyme, for example, or for a small molecule, and may be performed using any variety of methods, including those described or referred to herein.

[0197] In yet another aspect of the present invention, desirable existing compounds are modified, and evaluated for a more desirable compound. Existing compounds or compound libraries are exposed to molecules generated via the expression of small or large insert libraries generated in accordance with the methods described herein. Desirable modifications of these existing compounds by these molecules are detected and better lead compounds are screened for utilizing a fluorescence analyzer, such as a FACS machine. For example, E. coli cells expressing clones yielding small molecules are exposed to one or more existing compounds, which are subsequently screened for desirable modifications. Alternatively, cells are co-encapsulated with one or more existing compounds, and screened simultaneously to identify desirable modifications to the compound. Examples of modifications include covalent or non-covalent modifications. Covalent modifications include incorporation, transfer and cleavage modifications, such as the addition or transfer of methyl groups or phosphate groups to a compound, or the cleavage of a peptide or other bond to yield an active compound. Non-covalent modifications include conformational changes made to a molecule via addition or disruption of, for example, hydrogen bonds, ionic bonds, and/or Van der Wals forces. Modified compounds can be screened by various means, including those described herein.

[0198] Alternatively, existing compounds are utilized to modify the molecules generated via the expression of large or small insert clones, and desirable modifications of the molecules are screened for via fluorescence screening, utilizing various methods, including those described herein.

[0199] In another aspect of the present invention, molecules derived from expressed clones are exposed to organisms to enrich for potential compounds which cause growth inhibition or death of cells. For example, cultures of *Staphylococcus aureus* are co-encapsulated with compounds generated via expression of clones, or with cells expressing clones, and allowed to grow for a period of time by exposure to select media. Co-encapsulated products are then stained and screened for via fluorescence screening. Stains which allow detection of live cells can be utilized, allowing positives, which in this case would have no fluorescence, to be recovered. Alternatively, forward and side scatter characteristics are used to enrich for positives. Less or no growth of *Staphylococcus* or other organisms being evaluated will yield capsules with less forward and/or side scatter.

[0200] In another aspect of the present invention clones expressing useful bioactivities are screened in-vivo. In this aspect, host cells are stimulated to internalize recombinant cells, and used to screen for bioactivities generated by these recombinant cells which can cause host cell death or modify an internal molecule or compound within the host.

[0201] Many bacterial pathogens survive in phagocytes, such as macrophages, by coordinately regulating the expression of a wide spectrum of genes. A microbes ability to survive killing by phagocytes correlates with its ability to cause disease. Hence, the identification of genes that are preferentially transcribed in the intracellular environment of the host is central to understanding of how pathogenic organisms mount successful infection.

[0202] Valdivia and Falkow have reported a selection methodology to identify genes from pathogenic organisms that are induced upon association with host cells or

[illegible]

Questions have been asked about the effect of the tax credit on the number of children who are born to women who are poor.

[illegible]

Questions have been asked about the effect of the tax credit on the number of children who are adopted.

[illegible][illegible]

[0206] DNA isolation. DNA is isolated using the IsoQuick Procedure as per manufacturer's instructions (Orca, Research Inc., Bothell, WA). DNA can be normalized according to Example 2 below. Upon isolation the DNA is sheared by pushing and pulling the DNA through a 25G double-hub needle and a 1-cc syringes about 500 times. A small amount is run on a 0.8% agarose gel to make sure the majority of the DNA is in the desired size range (about 3-6 kb).

[0207] Blunt-ending DNA. The DNA is blunt-ended by mixing 45 µl of 10X Mung Bean Buffer, 2.0 µl Mung Bean Nuclease (150 u/µl) and water to a final volume of 405 µl. The mixture is incubate at 37⁰C for 15 minutes. The mixture is phenol/chloroform extracted followed by an additional chloroform extraction. One ml of ice cold ethanol is added to the final extract to precipitate the DNA. The DNA is precipitated for 10 minutes on ice. The DNA is removed by centrifugation in a microcentrifuge for 30 minutes. The pellet is washed with 1 ml of 70% ethanol and repelleted in the microcentrifuge. Following centrifugation the DNA is dried and gently resuspended in 26 µl of TE buffer.

[0208] Methylation of DNA. The DNA is methylated by mixing 4 µl of 10X *EcoR* I Methylase Buffer, 0.5 µl SAM (32 mM), 5.0 µl *EcoR* I Methylase (40 u/µl) and incubating at 37⁰C, 1 hour. In order to insure blunt ends, add to the methylation reaction: 5.0 µl of 100 mM MgCl₂, 8.0 µl of dNTP mix (2.5 mM of each dGTP, dATP, dTTP, dCTP), 4.0 µl of Klenow (5 u/µl) and incubate at 12⁰C for 30 minutes.

[0209] After 30 minutes add 450 µl 1X STE. The mixture is phenol/chloroform extracted once followed by an additional chloroform extraction. One ml of ice cold ethanol is added to the final extract to precipitate the DNA. The DNA is precipitated for 10 minutes on ice. The DNA is removed by centrifugation in a microcentrifuge for 30 minutes. The pellet is washed with 1 ml of 70% ethanol, repelleted in the microcentrifuge and allowed to dry for 10 minutes.

[0210] **Ligation.** The DNA is ligated by gently resuspending the DNA in 8 μ l EcoR I adaptors (from Stratagene's cDNA Synthesis Kit), 1.0 μ l of 10X Ligation Buffer, 1.0 μ l of 10 mM rATP, 1.0 μ l of T4 DNA Ligase (4Wu/ μ l) and incubating at 4°C for 2 days. The ligation reaction is terminated by heating for 30 minutes at 70°C.

[0211] **Phosphorylation of adaptors.** The adaptor ends are phosphorylated by mixing the ligation reaction with 1.0 μ l of 10X Ligation Buffer, 2.0 μ l of 10mM rATP, 6.0 μ l of H₂O, 1.0 μ l of polynucleotide kinase (PNK) and incubating at 37°C for 30 minutes. After 30 minutes 31 μ l H₂O and 5 ml 10X STE are added to the reaction and the sample is size fractionate on a Sephacryl S-500 spin column. The pooled fractions (1-3) are phenol/chloroform extracted once followed by an additional chloroform extraction. The DNA is precipitated by the addition of ice cold ethanol on ice for 10 minutes. The precipitate is pelleted by centrifugation in a microfuge at high speed for 30 minutes. The resulting pellet is washed with 1 ml 70% ethanol, repelleted by centrifugation and allowed to dry for 10 minutes. The sample is resuspended in 10.5 μ l TE buffer. Do not plate. Instead, ligate directly to lambda arms as above except use 2.5 μ l of DNA and no water.

[0212] **Sucrose Gradient (2.2 ml) Size Fractionation.** Stop ligation by heating the sample to 65°C for 10 minutes. Gently load sample on 2.2 ml sucrose gradient and centrifuge in mini-ultracentrifuge at 45K, 20°C for 4 hours (no brake). Collect fractions by puncturing the bottom of the gradient tube with a 20G needle and allowing the sucrose to flow through the needle. Collect the first 20 drops in a Falcon 2059 tube then collect 10 1-drop fractions (labeled 1-10). Each drop is about 60 μ l in volume. Run 5 μ l of each fraction on a 0.8% agarose gel to check the size. Pool fractions 1-4 (about 10-1.5 kb) and, in a separate tube, pool fractions 5-7 (about 5-0.5 kb). Add 1 ml ice cold ethanol to precipitate and place on ice for 10 minutes. Pellet the precipitate by centrifugation in a microfuge at high speed for 30 minutes. Wash the pellets by resuspending them in 1 ml 70% ethanol and repelleting them by centrifugation in a microfuge at high speed for 10 minutes and dry. Resuspend each pellet in 10 μ l of TE buffer.

[0213] **Test Ligation to Lambda Arms.** Plate assay by spotting 0.5 μ l of the sample on agarose containing ethidium bromide along with standards (DNA samples of known concentration) to get an approximate concentration. View the samples using UV light and estimate concentration compared to the standards. Fraction 1-4 = >1.0 μ g/ μ l. Fraction 5-7 = 500 ng/ μ l.

[0214] Prepare the following ligation reactions (5 μ l reactions) and incubate 4°C, overnight:

Sample	H ₂ O	10X Ligase Buffer	10mM rATP	Lambda arms (ZAP)	Insert DNA	T4 DN Ligase Wu/(l)
Fraction 1-4	0.5 μ l	0.5 μ l	0.5 μ l	1.0 μ l	2.0 μ l	0.5 μ l
Fraction 5-7	0.5 μ l	0.5 μ l	0.5 μ l	1.0 μ l	2.0 μ l	0.5 μ l

[0215] **Test Package and Plate.** Package the ligation reactions following manufacturer's protocol. Stop packaging reactions with 500 μ l SM buffer and pool packaging that came from the same ligation. Titer 1.0 μ l of each pooled reaction on appropriate host (OD₆₀₀ = 1.0) [XLI-Blue MRF]. Add 200 μ l host (in mM MgSO₄) to Falcon 2059 tubes, inoculate with 1 μ l packaged phage and incubate at 37°C for 15 minutes. Add about 3 ml 48°C top agar [50ml stock containing 150 μ l IPTG (0.5M) and 300 μ l X-GAL (350 mg/ml)] and plate on 100 mm plates. Incubate the plates at 37°C, overnight.

[0216] **Amplification of Libraries (5.0 x 10⁵ recombinants from each library).** Add 3.0 ml host cells (OD₆₀₀=1.0) to two 50 ml conical tube and inoculate with 2.5 X

10⁵ pfu of phage per conical tube. Incubate at 37°C for 20 minutes. Add top agar to each tube to a final volume of 45 ml. Plate each tube across five 150 mm plates. Incubate the plates at 37°C for 6-8 hours or until plaques are about pin-head in size. Overlay the plates with 8-10 ml SM Buffer and place at 4°C overnight (with gentle rocking if possible).

[0217] **Harvest Phage.** Recover phage suspension by pouring the SM buffer off each plate into a 50-ml conical tube. Add 3 ml of chloroform, shake vigorously and incubate at room temperature for 15 minutes. Centrifuge the tubes at 2K rpm for 10 minutes to remove cell debris. Pour supernatant into a sterile flask, add 500 µl chloroform and store at 4°C.

[0218] **Titer Amplified Library.** Make serial dilutions of the harvested phage (for example, 10⁻⁵ = 1 µl amplified phage in 1 ml SM Buffer; 10⁻⁶ = 1 µl of the 10⁻⁵ dilution in 1 ml SM Buffer). Add 200 µl host (in 10 mM MgSO₄) to two tubes. Inoculate one tube with 10 µl 10⁻⁶ dilution (10⁻⁵). Inoculate the other tube with 1 µl 10⁻⁶ dilution (10⁻⁶). Incubate at 37°C for 15 minutes. Add about 3 ml 48°C top agar [50ml stock containing 150 µl IPTG (0.5M) and 375 µl X-GAL (350 mg/ml)] to each tube and plate on 100 mm plates. Incubate the plates at 37°C, overnight.

[0219] Excise the ZAP II library to create the pBLUESCRIPT library according to manufacturers protocols (Stratagene).

Example 2

Normalization

[0220] Prior to library generation, purified DNA can be normalized. DNA is first fractionated according to the following protocol. A sample composed of genomic DNA is purified on a cesium-chloride gradient. The cesium chloride (R_f = 1.3980) solution is filtered through a 0.2 µm filter and 15 ml is loaded into a 35 ml OptiSeal tube (Beckman). The DNA is added and thoroughly mixed. Ten micrograms of

bis-benzimide (Sigma; Hoechst 33258) is added and mixed thoroughly. The tube is then filled with the filtered cesium chloride solution and spun in a VTi50 rotor in a Beckman L8-70 Ultracentrifuge at 33,000 rpm for 72 hours. Following centrifugation, a syringe pump and fractionator (Brandel Model 186) are used to drive the gradient through an ISCO UA-5 UV absorbance detector set to 280 nm. Peaks representing the DNA from the organisms present in an environmental sample are obtained. Eubacterial sequences can be detected by PCR amplification of DNA encoding rRNA from a 10-fold dilution of the *E. coli* peak using the following primers to amplify:

[0221] Forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3' SEQ ID NO: 1

[0222] Reverse primer: 5'-GGTACCTTGTTACGACTT-3' SEQ ID NO: 2

[0223] Recovered DNA is sheared or enzymatically digested to 3-6 kb fragments. Lone-linker primers are ligated and the DNA is size selected. Size-selected DNA is amplified by PCR, if necessary.

[0224] Normalization is then accomplished as follows by resuspending double-stranded DNA sample in hybridization buffer (0.12 M NaH₂PO₄, pH 6.8/0.82 M NaCl/1 mM EDTA/0.1% SDS). The sample is overlaid with mineral oil and denatured by boiling for 10 minutes. Sample is incubated at 68°C for 12-36 hours. Double-stranded DNA is separated from single-stranded DNA according to standard protocols (Sambrook, 1989) on hydroxyapatite at 60°C. The single-stranded DNA fraction is desalted and amplified by PCR. The process is repeated for several more rounds (up to 5 or more).

Example 3

Cell Staining Prior to FACS Screening

[0225] Gene libraries, including those generated as described in Example 1, can be screened for bioactivities of interest on a FACS machine as indicated herein. A

screening process begins with staining of the cells with a desirable substrate according to the following example.

[0226] A gene library is made from the hyperthermophilic archaeon *Sulfolobus solfataricus* in the λ -ZAPII vector according to the manufacturers instructions (Stratagene Cloning Systems, Inc., La Jolla, CA), and excised into the pBLUESCRIPT plasmid according to the manufacturers instructions (Stratagene). DNA was isolated using the IsoQuick DNA isolation kit according to the manufacturers instructions (Orca, Inc., Bothell, WA).

[0227] To screen for β -galactosidase activity, cells are stained as follows. Cells are cultivated overnight at 37°C in an orbital shaker at 250rpm. Cells are centrifuged to collect about 2×10^7 cells (0.1ml of the culture), resuspended in 1ml of deionized water, and stained with C₁₂-Fluorescein-Di-(-D-galactopyranoside (FDG). Briefly, 0.5ml of cells are mixed with 50 μ l C₁₂-FDG staining solution (1mg C₁₂-FDG in 1ml of a mixture of 98% H₂O, 1% DMSO, 1% EtOH) and 50 μ l Propidium iodide (PI) staining solution (50 μ g/ml of distilled water). The sample is incubated in the dark at 37°C with shaking at 150rpm for 30 minutes. Cells are then heated to 70°C for 30 minutes (this step can be avoided if sample is not derived from a hyperthermophilic organism).

Example 4

Screening of Expression Libraries by FACS and Recovery of Genetic Information of Sorted Organisms

[0228] The excised λ -ZAP II library is incubated for 2 hours and induced with IPTG. Cells are centrifuged, washed and stained with the desired enzyme substrate, for example C₁₂-Fluorescein-Di-(-D-galactopyranoside (FDG) as in Example 3. Clones are sorted on a commercially available FACS machine, and positives are collected. Cells are lysed according to standard techniques (Current Protocols in Molecular Biology, 1987) and plasmids are transformed into new host by electroporation using standard techniques. Transformed cells are plated for secondary

screening. The procedure is illustrated in Figure 5. Sorted organisms can be grown and plated for secondary screening.

Example 5

Sorting Directly on Microtiter Plates

[0229] Cells can be sorted in a FACS instrument directly on microtiter plates in accordance with the present invention. Sorting in this fashion facilitates downstream processing of positive clones.

[0230] *E.coli* cells containing β -galactosidase genes are exposed to a staining solution in accordance with Example 3. These cells are then left to sit on ice for three minutes. For the cell sorting procedure they are diluted 1:100 in deionized water or in Phosphate Buffered Saline solution according to the manufacturers protocols for cell sorting. The cells are then sorted by the FACS instrument into microtiter plates, one cell per well. The sorting criteria is fluorescein fluorescence indicating β -galactosidase activity or PI for indicating the staining of dead cells (unlike viable cells, dead cells have no membrane potential; hence PI remains in the cell with dead cells and is pumped out with live cells). Results as observed on the microtiter plate are shown in Figure 6.

[0231] **Table 1**

<u>Habitat</u>	<u>Cultured (%)</u>
Seawater	0.001-0.1
Freshwater	0.25
Mesotrophic lake	0.01-1.0
Unpolluted esturine waters	0.1-3.0
Activated sludge	1.0-15.0

Sediments	0.25
Soil	0.3

Example 6

Production of single cells or fragmented mycelia

[0232] Inoculate 25ml MYME media (see recipe below) in 250 ml baffled flask with 100 µl of *Streptomyces* 10712 spore suspension and incubated overnight @ 30°C 250rpm. After 24 hour incubation, transfer 10ml to 50ml conical polypropylene centrifuge tube and centrifuge @ 4,000rpm for 10 minutes @ 25°C. Decant supernatant and resuspend pellet in 10ml 0.05M TES buffer. Sort cells into MYM agar plates (sort 1 cell per drop, 5 cells per drop, 10 cells per drop) and incubate plates @ 30°C.

[0233] MYME media (Yang, et.al., 1995 J. Bacteriol. 177(21): 6111-6117) contains: 10.3% sucrose, 1% maltose, 0.5% peptone, 0.3% yeast extract, 0.3% maltose extract, 5mM MgCl₂ and 1% glycine

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[0239] It will be apparent to those skilled in the art that various modifications and variations can be made to the compounds and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents. Accordingly, the invention is limited only by the following claims.